We rationally designed, synthesized and tested a novel set of ligands to exploit a hydrophobic cavity in the vicinity of the docking pose of known ligands. All novel ligands showed improved, affinities with respect to their reference compounds, in good quantitative agreement between experiment and simulation, some of them reaching nanomolar affinities. Studying a series of site-specific mutations, we could computationally rationalize the receptor selectivity of specific compounds to either preferentially bind CB1 or CB2, respectively.

[1] S.H. Burstein and R.B. Zurier (2009) Cannabinoids, Endocannabinoids, and Related Analogs in Inflammation,
AAPS J. 11(1), 109-119.
[2] H. Merlitz, B. Burghardt and W. Wenzel (2003) Application of the Stochastic Tunneling Method to High Throughput Database Screening,
Chem. Phys. Lett. 370, 68-73.

2984-Pos Board B89

Field-Modulated Magnetic Birefringence Relaxation to Assay b-Adrenergic Receptor-Ligand Association

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We developed a platform for monitoring the association between biologically active molecules and their cognate receptors based on measuring rate changes of magnetic birefringence relaxation of superparamagnetic nanoparticles. The method is particularly designed for rapid screening of display libraries and mutant receptor libraries expressed in bacteria and yeast. The magnitude of birefringence rate change after particle orientation in a magnetic field reflects the difference of nanoparticle hydrodynamic volume upon formation of the ''receptor-ligand complex''. The rationale for using nanoparticle birefringence to identify ligand and receptor interactions is that it eliminates the need for removing unbound ligands. We have previously demonstrated that maghemite nanoparticles, decorated with ligand molecules, exhibit longer relaxation times when associated with proteins, viruses and small size microorganisms. We utilize magnetic field-modulated birefringence to monitor association between a ligand (alprenolol) immobilized on the surface of magnetic nanoparticles and β-adrenergic receptor overexpressed on the cell surface of bacteria and yeast. Upon association of the ligand with the receptor, we observed an increase in the birefringence rate constant, an effect that was fully reversible by adding β-adrenergic competitor ligands. Rate constant distributions were reconstructed using the Phase Function Method. We show that birefringence signals can be used to monitor the dynamics of particle rotational alignment as well as rotational relaxation. Alignment dynamics are characteristic of the number of accessible receptor sites while relaxation characterizes the aggregate size. This novel approach is applicable in systems where the interaction of ligand with cognate receptor does not activate intracellular pathways and thus are difficult to monitor with a ratiofluorometric platform. This method will be useful for a broad range of applications, including discovery research to develop small molecule drugs and for identification of orphan receptors and biologically active peptides and proteins from display libraries.

2985-Pos Board B90

Transient Kinetics of Nucleotide Binding to the P-glycoprotein Multidrug Transporter

Miguel R. Lugo, Joseph W.K. Chu, Frances J. Sharom.

The P-glycoprotein multidrug transporter (Pgp) is a member of the ABC (ATP-binding cassette) superfamily of proteins, and contains two highly conserved cytosolic nucleotide-binding domains (NBDs). These domains couple ATP hydrolysis to the export of a variety of hydrophobic natural products, chemotherapeutic drugs and peptides. Pgp ATPase activity has been extensively characterized, but many details of the catalytic cycle of ATP hydrolysis remain unexplored. The fluorescent nucleotide analogue, TNP-ADP, interacts with the NBDs of Pgp, and the stoichiometry and affinity of binding were previously determined in our laboratory. Transient kinetic methods are commonly used to investigate the mechanism of molecular processes over a time-scale from milliseconds to hundreds of seconds. In this work, we studied the binding of TNP-ADP to Pgp using rapid stopped-flow kinetics. In these experiments, the binding reaction was monitored by following different fluorescent signals, under pseudo-first-order conditions (excess ligand). The time course of TNP-ADP binding displayed five relaxation times (τ_i's) over a time-span of 300 sec, which correspond to at least five different transitions. All the relaxation times presented a strong temperature-dependence. The time course of the reaction was analyzed by the computational tool Global Kinetic Explorer (KinTek, USA), using several models of sequential isomerizations after/before the fast bimolecular binding reaction. Also, the parameters were analyzed using the matrix projection operator technique for kinetic data (Bujalowski *et al.*, 2000, J. Mol. Biol. 295:831). Both approaches provide, for the first time, information about the rate constants and fluorescent properties of the diverse intermediates formed during binding of TNP-ADP to Pgp. Elucidation of the details of the interaction of nucleotides with Pgp is of prime importance for formulation of a detailed mechanism of action of the transporter.

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2986-Pos Board B91

Identification of an Efficacy Pharmacophore for μ Opioid Receptor Ligands Using the Conformationally Sampled Pharmacophore (CSP) Method

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μ opioid receptor agonists and antagonists play a critical role in the treatment of severe pain and as treatments for drug abuse. Their structure activity relationships (SAR) have been extensively investigated followed by lead optimization. However, challenges remain in improving the utility of m ligands with respect to reducing adverse effects such as tolerance, dependence, constipation, etc. while maintaining the potency of current medicines. To facilitate the meeting of these challenges, consensus pharmacophore models for diverse classes of μ opioid receptor ligands were established and we constructed predictive model differentiating agonists and antagonists activities using the conformationally sampled pharmacophore (CSP) method. The predictability of the models was validated against a number of classes of opioids including 4,5-Epoxymorphinans, Diels-Alder adducts of thebaine, Benzomorphans, Methadone, Fentanyl, and, notably, non-nitrogenous opioids; a collection of compounds which have eluded a consensus SAR of opioids for decades. The consensus model was derived by virtue of CSP method incorporating flexibility of molecules through using all-accessible conformers in model development and by eliminating any limitations associated with alignment procedures. The procedures to develop and features of the CSP model will be presented.

2987-Pos Board B92

Probing Binding of Apelin to the Extracellular Loops of the Apelin Receptor (APJ) in Lipid Mimetic Environments

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The apelin receptor, previously called APJ, is a G protein-coupled receptor highly expressed in the central nervous system (CNS), cardiovascular system, the adipoinsular axis and the mammary glands, among other tissues and organ systems. The apelinergic system plays important biological functions in the regulation of blood pressure, blood glucose, drinking behavior and food intake. The action of this system is also implicated in tumour angiogenesis, diabetes and cardiovascular diseases. In addition, the apelin receptor is a co-receptor for human immunodeficiency virus type 1 (HIV1) and simian immunodeficiency virus (SIV). Despite these roles, the mechanism of activation of apelin receptor by its cognate ligand has not been studied at the molecular level. Following the ''divide and conquer'' approach for membrane protein characterization, we present the biophysical characterization of the interaction of two extracellular loops of the apelin receptor (extracellular loops 1, EL1 and 3, EL3) with a fluorescently-tagged apelin analogue in lipid environment. Peptides were synthesized by solid phase peptide synthesis and purified by high-performance liquid chromatography. Characterization both of the peptides in isolation and of the binding between apelin and EL peptides is provided using circular dichroism spectroscopy, fluorescence resonance energy transfer and nuclear magnetic resonance spectroscopy. These results provide insight into understanding of the apelinergic system at the molecular level and provide the first structural information on the apelin receptor for the development of therapeutics targeting this system.

2988-Pos Board B93

Design and Biophysical Characterization of a Single Chain Four-alpha-helix Bundle Protein Which Binds Volatile General Anesthetics

Lucia M. Morstadt, Qing Cheng Meng, Jonas S. Johansson.

In order to investigate the interaction of volatile general anesthetics with their putative membrane protein targets, we designed a four-alpha-helix bundle protein in a single protein chain. The four alpha helices, connected by three 8 glycine loops, had the sequence A, B, B', A'. The DNA sequence was designed with the goal of making helices with the same amino acid sequence (helix A and A', B and B', respectively) as different as possible in their

DNA sequence, while using codons which are favorable for expression in E.coli. Restriction enzyme sites were added on both ends. The synthesized DNA sequence was cloned into an expression vector. The protein was bacterially expressed and purified to homogeneity using reverse-phase HPLC. Protein identity was verified using MALDI-TOF mass spectroscopy. Near UV circular dichroism spectroscopy confirmed the strongly alpha helical nature of the protein. Guanidinuium chloride denaturation showed that the single chain four-alpha-helix bundle protein is twice as stable as the dimeric dihelical protein. The sigmoidal character of the unfolding reaction was conserved, the sharpness of the transition increased. Our single chain four-alpha-helix bundle protein bound halothane with a dissociation constant of 1.2 mM, as shown by tryptophan fluorescence quenching. This single chain four-alpha-helix bundle protein can now be used as a scaffold to incorporate natural membrane protein sequences to examine general anesthetic interactions in detail.

2989-Pos Board B94

Binding Profiles Based on Normal Mode Analysis as a Foundation for a Unified Approach to Allosteric Activation of Prolactin Receptor

Adam D. Schuyler.

Two of the most fundamental biological processes are ligand binding and allosteric signaling. Despite their (often) direct linkage, a unified model of the underlying dynamics is not well established. The harmonic motions identified by normal mode analysis (NMA) provide a natural coordinate system for conformation space. Binding profiles and allosteric profiles based on NMA allow for a unified model which describes the propensity for various structure motions to promote/inhibit binding and allosteric activation. The authors have recently developed the allosteric model; the current work presents the binding model.

The normal modes describe local motions accessible to a conformation. Each of the modes is followed over a small displacement, leading to a set of new conformations. This procedure is repeated to generate an ensemble, where each conformation is defined by its sequence of normal modes. Ensembles are generated around a receptor and ligand. Each receptor conformation is paired against each ligand conformation and scored for: (1) compatibility of interface shape; and (2) compatibility of interface dynamics. These scores are attributed to the normal mode sequences associated with each candidate conformation. The scores are summed over all possible conformation pairings, producing a binding profile that defines each normal mode's propensity to contribute to compatible interface shape and interface dynamics.

The prolactin receptor dimerizes and preferentially binds prolactin at one binding site, and then binds another prolactin at the second binding site - activating the receptor. Binding profiles are generated for prolactin and prolactin receptor, isolated from the various complexes along the activation pathway. The goal is to identify the dynamics that regulate binding site preference and allosteric activation. Future work will incorporate NMR studies to validate and refine the initial results.

2990-Pos Board B95

Solid-State NMR Study of Ligand Binding to Human Peripheral Cannabinoid Receptor CB2

Tomohiro Kimura, Alexei Yeliseev, Mihaela Mihailescu, Kejun Cheng, Kenner C. Rice, Klaus Gawrisch.

The peripheral cannabinoid receptor CB2 belongs to the family of G protein coupled receptors (GPCRs). Ligand binding studies on CB2 are complicated by high affinity of the endogenous or exogenous cannabinoid ligands for the lipid matrix that hosts the GPCR. Here we show that solid-state NMR distinguishes between specific ligand-binding to CB2 and nonspecific interactions with the lipid matrix. Experiments were conducted with recombinant, human CB2 expressed in *E. coli*, purified and functionally reconstituted into unilamellar liposomes. Location, structure, and dynamics of ligands in the lipid matrix were probed by NMR as well as neutron diffraction. The synthetic agonist CP-55,940 locates near the hydrophobic/hydrophilic interface of bilayers with its bond linking hydroxyphenyl and hydroxycyclohexyl rings perpendicular to the bilayer normal, while the endogenous agonist 2-AG orients parallel to the bilayer normal with the glycerol moiety near the hydrophobic/hydrophilic interface and the arachidonoyl chain in the hydrophobic region. Both ligands maintain a high level of conformational flexibility and have lateral diffusion rates in membranes comparable to those of lipids. Ligand binding to CB2 drastically shortens transverse relaxation times of the ^{2}H-labeled ligands which distinguish between specific and non-specific binding events. Competition binding experiments with protonated and selectively deuterated CP-55,940 showed that ~90% of the reconstituted CB2 was ligand-binding competent and formed a one-to-one complex with the ligand. Activation of G protein by agonist-bound CB2 was confirmed in a G protein activation assay. The endogenous 2-AG has a binding affinity to CB2 that is orders of magnitude lower compared to CP-55,940. The possibility that cannabinoid ligands approach the receptor from the lipid matrix will be discussed. Experiments are underway to gain structural insights into specific binding interactions based on selective isotope-labeling of both ligand and recombinant CB2.

2991-Pos Board B96

Fluorescence Guided Force Microscopy (FGFM) Used to Measure Receptor Ligand Interactions in Live Mammalian Cells

Jeremy C. Bonor.

Fluorescence Guided Force Microscopy (FGFM) is a new technique that was developed in order to measure and quantify ligand surface interactions on the cell surface in living cells. Using this method we can determine the binding affinity of ligands with surface receptors and their spatial distribution by combining force volume measurements, confocal microscopy and atomic force microscopy into one new instrumental setup. In order to validate this new method we covalently linked a ligand, Bone Morphogenetic Protein 2 (BMP2) to an Atom Force Microscopy (AFM) probe and acquired topographical and force binding information. To visualize caveolae by confocal microscopy, membrane domains known to co-localize with BMP receptors on the cell surface, Caveolin-1 was fused to green or red fluorescent protein and used as a marker. We transfected C2C12 cells with plasmids encoding Caveolin-1 isoforms alpha and beta (fused to green fluorescent protein) and Caveolin-1 alpha (fused to red fluorescent protein). Detecting the unbinding forces, surface topography and fluorescent protein location on the live cell surface, required combining Force Volume measurements, AFM and confocal imaging; which we achieved through the integration of the Veeco Bioscope2 AFM module, equipped with a closed loop scanner and a Zeiss LSM510 NLO confocal. The collection of high resolution confocal images, AFM images and unbinding force curve data on live cells allowed us to resolve the spatial distribution of binding events on the plasma membrane of C2C12 cells. This data showed that BMP 2 bound with the highest affinity inside Caveolin-1 isoforms.

2992-Pos Board B97

Nanoparticles Masquerade as "Self" to Inhibit Phagocytosis

Pia L. Rodriguez, Takamasa Harada, Dennis E. Discher.

A major challenge for injecting particles or implanting biomaterials into the body is that they activate immune cells such as macrophages, the cells that normally function to clear invading pathogens. Interestingly, macrophages have a surface receptor mechanism which prevents them from phagocytosing our own ''self'' cells. During initial macrophage engulfment, macrophages recognize foreign and self targets because they both have antibodies or plasma complement proteins on their surface. However, before the macrophage engulfs the target, self cells are checked for the presence of the surface protein CD47 which will bind to the macrophage receptor SIRPα (CD172), and we show that CD47-SIRPα interactions in cell-cell adhesion, with human macrophages in sparse culture, produce phagocytosis inhibition. Whether the CD47 interaction is functional with small targets of phagocytosis is unclear and relevant perhaps to nano-sized targets. We show that CD47 coupled to a series of synthetic beads can inhibit uptake by macrophages. However, we need to test this interaction and observe how these results are consistent with in vivo systems. We are currently testing whether nanoparticles that have surface immobilized hCD47 or a portion of it will not be phagocytosed in nobese diabetic (NOD) mice, we believe that hCD47 will bind to this mSirpa strain and that could bring a better understanding of the interaction at a nanoscale.

2993-Pos Board B98

In Vivo Binding Kinetics and Stoichiometry of Toll-Like Receptor 9 and CpG DNA Resolved by Multiparametric Single Molecule Techniques

Jiji Chen, Suman Nag, Joseph Irudayaraj.

Toll-like receptor 9 (TLR9) activates the innate immune system in response to oligonucleotides rich in CpG whereas DNA lacking CpG could inhibit its activation. Although in vitro experiments demonstrate TLR9 binding to nucleic acids, the mechanism of how this receptor interacts with nucleic acid and becomes activated in live cells is far from behind understood. Here, we report on the successful implementation of single molecule tools, constituting fluorescence correlation spectroscopy (FCS), fluorescence cross-correlation spectroscopy (FCCS), photon count histogram (PCH) and fluorescence lifetime imaging (FLIM) to study the interaction of TLR9-GFP with Cy5 labeled oligonucleotide containing CpG or lacking CpG in live cells.